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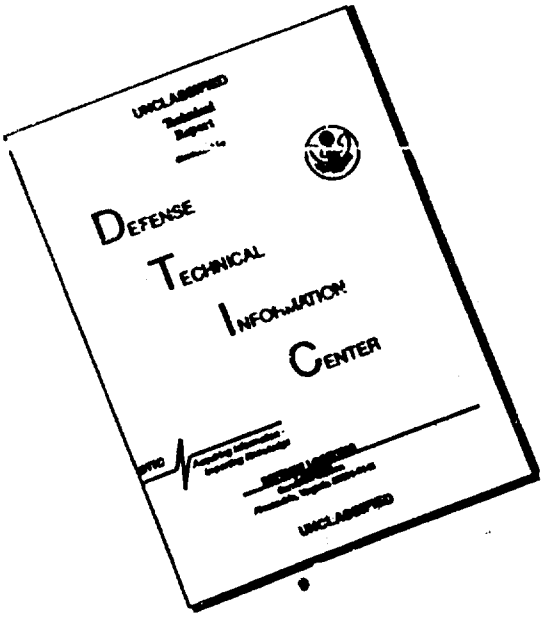

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Use of the Indirect Hemagglutination Reaction in Determination of Botulinal Toxin in Industrial Sausage Manufacture

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The detection of botulinal toxin in foodstuffs is of great importance for the prevention of botulism. Actually, the only reliable and practically the only available method of diagnosis is the biological test on white mice, or guinea pigs. By now, numerous improvements in the biological test have been proposed and they permit us to speed up considerably the time of detection, but, if we deal with a low concentration of toxin in daily food products, still 14 to 18, or even more hours are required for the discovery of toxin. Various methods in vitro (MINERVIN et al.) suggested for detection of botulinal toxin so far failed to find a general acceptance due to cumbersome research work and unreliability of the results obtained.

For the purpose of determination of botulinal toxin in food products, RYTSAI (RYCAJ - correctly, proposed quite recently a modified reaction of the indirect hemagglutination and, thus, he

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raised again the question of advisability of the reaction in vitro. According to his opinion, erythrocytes are not sensitized with antigen, but with antibodies. Moreover, he used processed erythrocytes with tannic acid in a tenfold concentration, as compared with the universally adopted concentration (1:2,000); he also made certain changes in the pH of salt solutions used in the reaction and in the concentration of the normal serum added. According to the findings of RYTSAI, the indirect hemagglutination reaction (in his modification) is even more sensitive than the biological test.

Having reproduced exactly the method of RYTSAI, we obtained negative results from the indirect hemagglutination reaction with botulinal toxin. In connection with this, certain changes were introduced to the procedure of the reaction and they were fully confirmative. It should be noted that two reports were published in the Zhurn. Mikrobiologii, Epidemiologii i Immunologii, No.3 and 4, 1960. One is that of SINITSYN, who checked the suggestions of RYTSAI and obtained encouraging results. SINITSYN introduced a series of changes to the reaction and thus increased its sensitivity. The report of SINITSYN was published already after the completion of our experiments and we found that it contained somewhat different technical recommendations in comparison with ours.

In the first series of experiments we performed a preparatory processing (sensitization) of tanned erythrocytes with toxins in various concentrations. Evidently, the variant used was unsuitable, thus we were compelled (as RYTSAI suggested) to try the indirect hemagglutination reaction with erythrocytes previously processed

with antibotulinal sera of the A and B types. We tested in these experiments various series of therapeutic and diagnostic[1] antitoxic sera. We always obtained negative results from the indirect hemagglutination reaction while using unpurified sera in spite of the presence of some obvious preventative factor in the biological test. Therefore, we used only therapeutic sera purified by the Diaferm-3 methods (MECHNIKOV'S Institute of Vaccines and Sera, Moscow) and they proved fully satisfactory. Apparently, the negative results obtained with crude antitoxic sera can be explained by the presence of albumin in the sera, which, as is well known, exerts an inhibitory effect on the indirect hemagglutination reaction (NETER et al.).

We conducted our experiments with botulinal toxins type A and B of different series and in various concentrations.

In accordance with the final technique in detection of botulinal toxin by way of the hemagglutination reaction, we mixed in a physiological solution in equal proportions a 2.5% suspension of sheep erythrocytes (washed until hemolytic fluid disappeared completely) with a tannin solution (1:20,000); then, we kept this for 10 minutes at 37°C. Subsequently, we washed erythrocytes free of tannic acid once, using physiological solution. Two batches of 0.1 ml each of the washed erythrocytes were saturated with 3 ml of therapeutic antibotulinal sera of the A and B types respectively (concentration 1:10) in a phosphate buffered physiological solution (pH=6.4); the

1) - The latter were kindly offered by K.I.MATVEEV and T.I. BULATOVA (GAMALEI'S Institute of Epidemiology and Microbiology, Academy of Medical Sciences, USSR)

type A serum contained 7250 BU in 1 ml and serum B 2500 BU. The saturation was accomplished at 37°C in 50 minutes, whereupon erythrocytes were washed twice in a physiological solution (nonbuffered) containing 1% inactivated normal rabbit serum (inactivation effected in 30 minutes in a water bath at 56°C). From the processed and washed erythrocytes a 2% suspension was prepared in a phosphate buffered physiological solution (pH=7.0) with 0.4% of normal rabbit serum added. Then, 0.1 ml portion of the mentioned suspension was added to each test tube containing the toxin. Twofold dilutions of the latter were prepared in advance in series beginning with 1:1,000 up to 1:10,000 in a phosphate buffered physiological solution (pH=7.0) with 0.4% of normal rabbit serum added. Having placed in test tubes 0.5 ml of the toxin of each dilution, the contents of the test tubes was agitated and stored at 37°C for 60 minutes. The checking of the results followed immediately after. It is necessary to fulfill exactly the recommendations concerning the use of buffered and nonbuffered physiological solutions, also the pH level and the concentration of normal rabbit serum that prevents the spontaneous agglutination of erythrocytes after processing with tannic acid.

The results of the experiments proved a high sensitivity of the indirect hemagglutination reaction. Thus, we succeeded in determination of the toxin type A in a concentration of 1:320,000 (Dlm for white mice 1:500,000). At the same time we found that erythrocytes processed with serum B were also agglutinated by the type A toxin, although to a lesser degree (Table 1).

Table 1

Reaction of Indirect Hemagglutination with Botuline Toxin Type A

Serum	Toxin's concentration										Control
	1:10 000	1:20 000	1:40 000	1:80 000	1:160 000	1:320 000	1:640 000	1:1 280 000	1:2 560 000	1:5 120 000	
A	+++	+++	+++	+++	+++	+++	+	-	-	-	-
B	+++	+++	++	++	-	-	-	-	-	-	-

Symbols: +++, ++, +, intensity of positive reactions; — negative reactions.

As we know, the botulinal toxin can evoke a nonspecific agglutination with nonsensitized erythrocytes (LAMANNA). Thus, it can be assumed that cross reactions are specifically caused by this circumstance: the agglutination effect develops in the presence of high concentrations of toxin and, as the concentration decreases, the effect gradually disappears. RYTSAI and later SINITSYN recommended that, in order to prevent a side nonspecific hemagglutination effect of botulinal toxin, one has to process (deplete) the toxins with erythrocytes beforehand. Apparently, this recommendation is based on erroneous analogies with various immunological reactions calculated to saturate immune agglutinating sera. An experimental test with a prior depletion of toxins with erythrocytes proved that no simultaneous changes occurred in the character of the subsequent indirect hemagglutination reaction, i.e. the depleted and the usual toxins showed quite alike behavior (Table 2).

In order to prevent cross reactions between the toxins of the A and B types, we accomplished a depletion of the therapeutic antitoxic sera with erythrocytes to which we previously adsorbed toxins of heterologous types. But, this method also proved unsuccessful and the cross reactions still persisted. Evidently, they are linked either with the presence of general antigens in toxins of the A and B types, or with the familiar polyvalency of the industrially produced sera. Our efforts to eliminate the polyvalency of the sera proved unsuccessful.

With the progress of our experiments we tried to establish the possibility of using the indirect hemagglutination reaction for

Table 2

Reaction of Indirect Hemagglutination with Botuline Toxin Depleted and not Depleted by Erythrocytes

Serum	Type of toxin	Form of toxin	Toxin's concentration										Control
			Whole	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	
B	A	Depleted	+++	++	-	-	-	-	-	-	-	-	-
		Not depleted	+++	++	-	-	-	-	-	-	-	-	-
	P	Depleted	+++	++	+++	+++	++	-	-	-	-	-	-
		Not depleted	+++	+++	+++	+++	+++	-	-	-	-	-	-

Symbols the same as in table 1.

Remark: toxin 1:1000 denoted as "whole" was used for depletion.

detection of botulinal toxin in industrial sausage manufacture. We used for this purpose a 5 gm portion of the product, which we cut into small pieces and, placing them in a small mortar, we poured over them 3 ml of botulinal toxin, either type A or B (we used toxins' series of different strength in our experiments) and left standing at room temperature for 5 minutes. Next, we added to each mortar 10 to 20 ml of phosphate buffered physiological solution (pH=7.0) with 0,4% of normal rabbit serum and we triturated this carefully. Then, we centrifuged the obtained suspension at 2,000 revolutions per minute for 5 to 10 minutes. We used the supernatant fluid (extract) in 0.5 ml volumes for the indirect hemagglutination reaction. Then. we used for control purposes a pure toxin in approximately the same concentration (allowing for the volume of fluid used to obtain the extract) and also an extract of sausage uninfected.

In addition to the direct infection of sausages with botulinal toxins types A and B, we also infected sausages with a 5-day culture of Cl. botulinum type A (strain No.98) and B (strain No.255). After the infection, sausages were stored in an incubator at 37°C for 7 days and then for 1½ to 2 weeks at 8 to 10°C. In these instances the extract for the indirect hemagglutination reaction was prepared according to the aforedescribed method.

The same extract was administered to white mice intraabdominally in 1 ml doses for control purposes. During 30 minutes prior to administration of the extract, some white mice were administered subcutaneously type A and B sera (500 BU each). The observation of in-

Table 3

Determination of Toxin in Boiled Sausage Following Infection With Botuline Toxin

Type of administered toxin	Type of serum	Minimal concentration of extract at which a detection of toxin occurred	
		Reaction of indirect hemagglutination	Biological test
A	A	1.16	1:50
	B	1:4	Negative
B	A	1:4	Whole extract
	B	1:8	Whole extract
—	A	Negative	Negative
	B	Negative	Negative

Table 4

Determination of Toxin in a Half-Smoked Sausage Following Infection With Spores of Cl. Botulinum Types A and B

Type of administered culture	Type of serum	Minimal concentration of extract at which a detection of toxin occurred	
		Reaction of indirect hemagglutination	Biological test
A	A	1:64	Whole extract
	B	1:32	Negative
B	A	1:8	Negative
	B	1:8	Negative

fected animals lasted 5 days.

Type A toxin was detected with the aid of the indirect hemagglutination reaction even after the extract had been diluted 16 times (Table 3); the reaction proved positive also with erythrocytes that were sensitized with the serum type B (1:4 concentration of the extract).

In other words, also in these experiments we failed to obtain a clear differentiation of the A and B types of botulinal toxin by means of the indirect hemagglutination reaction. At the same time, the biological test on white mice conducted simultaneously with the indirect hemagglutination reaction permitted us to detect type A toxin when the extract was diluted 50 times; thus, the biological test proved to be more sensitive in this case. Furthermore, we also succeeded in differentiation of the type of toxin, because the type B serum did not exert a preventative effect. We detected type B toxin by the indirect hemagglutination reaction when the concentration of the extract was 1:8 with the serum type B, and 1:4 with the serum type A. It should be mentioned that although the indirect hemagglutination reaction did not permit us (also in this case) to differentiate clearly the type of toxin, yet it proved to be more sensitive than the biological test: death of animals, which had not received a serum injection, resulted only after infection with whole extract. We also failed to obtain a differentiation of the type B toxin by means of the biological test.

Control tests with the extract from not contaminated sausage proved the absence of toxin in the indirect hemagglutination reaction

and in the biological test.

In investigation of the sausage infected with cultures of Cl. botulinum types A and B, the indirect hemagglutination reaction was more sensitive than the biological test (Table 4). Apparently, a partial (A) or complete (B) destruction of the toxin occurred during storage of the product, although the antigen components that participated in the indirect hemagglutination reaction were preserved. The above data prove that the indirect hemagglutination reaction can be used for retrospective diagnosis after the biological test has already produced negative results.

Conclusions

1. The indirect hemagglutination reaction was sufficiently sensitive and it made possible to detect botulinal toxin in sausage products within 3 hours, while no less than 18 to 24 hours are required to accomplish this with the biological test.

2. The indirect hemagglutination reaction is specific, although it is practically impossible to effect the differentiation of the A and B types of botulinal toxins by means of the industrially produced sera.

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Summary (copied)

Data are presented on the use of the indirect hemagglutination reaction for determination of botulism in sausages. The reaction proved to be sufficiently sensitive (in practice it was as sensitive as the biological test on animals); however, it was impossible to differentiate botulins of the A and B types with the aid of the existing sera. Indirect hemagglutination reaction may evidently be used for retrospective diagnosis when the biological test may have already given a negative result.